



Hammann, S., Korf, A., Bull, I. D., Hayen, H., & Cramp, L. J. E. (2019). Lipid profiling and analytical discrimination of seven cereals using high temperature gas chromatography coupled to high resolution quadrupole time-of-flight mass spectrometry. *Food Chemistry*, 282, 27-35.
<https://doi.org/10.1016/j.foodchem.2018.12.109>

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[10.1016/j.foodchem.2018.12.109](https://doi.org/10.1016/j.foodchem.2018.12.109)

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Lipid profiling and analytical discrimination of seven cereals using high temperature gas chromatography coupled to high resolution quadrupole time-of-flight mass spectrometry

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Abstract

Minor lipids in cereals (such as phytosterols and alkylresorcinols) can be important for human nutrition and/or be used as biomarkers for cereal intake. However, the analysis of cereal lipids is very challenging due to the complex lipidome comprising several hundred individual compounds present over a wide-range of concentrations.

Here we present a method for the profiling of cereal lipids using high temperature gas chromatography coupled to high resolution mass spectrometry (GC/Q-TOF MS). The method was used to investigate the lipid profiles of 77 samples of bread wheat, spelt, einkorn, emmer, barley, rye and oats.

Distinct differences in the patterns of alkylresorcinols, free and conjugated sterols and tocopherols between the cereals could be observed. Furthermore, traces of tocomonoenols and diunsaturated and methyl-alkylresorcinols (not previously reported in cereals) could be detected. Finally, the lipid patterns in the cereals could be used to separate the cereals by Principle Component Analysis.

Keywords: Cereal; Gas chromatography; Mass spectrometry; lipid; sterol; alkylresorcinol; tocopherol; Principle Component Analysis

1. Introduction

Although cereals have only been a part of human diet for roughly 10 000 years, they nowadays are a staple in most parts of the world. In 2017, the global production of cereals exceeded 2.5 billion tonnes, with wheat and rice comprising almost half (Food and Agriculture Organisation of the United Nations, 2018). Cereals are usually the primary dietary source of carbohydrates and therefore important for their calorific value, but they also contain proteins and lipids at lower concentrations, as well as several essential vitamins (McKevith, 2004).

Cereal lipids comprise a very complex mixture of compounds that are distributed in the cereal grain. A common concept is their classification as either “starch lipids” or “non-starch lipids” (Morrison, 1988) based on their association with starch granules in the cereal. An alternative categorisation, based on molecular structure, leads to classifications such as “polar” and “non-polar lipids”, with glycerophospholipids or mono- and digalactosyldiacylglycerols being the most prominent members of the former category. Non-polar lipids include triacylglycerols, sterols and sterol esters and tocopherols. However, it should be noted that there are no strict rules for classification.

Several minor compounds in the lipid fraction have attracted attention due to the health benefits for humans associated with them. Plant sterols and their conjugates (Figure 1a-d), as found in cereals, can lower the plasma cholesterol levels in humans and thus lower the risk of cardiovascular diseases. Similarly, tocopherols (Vitamin E) (Figure 1e-g) and alkylresorcinols (Figure 1h) are considered to be valuable dietary antioxidants and anticarcinogens that can help to prevent cell damage *in vitro* (Panfili,

Fратиanni, & Irano, 2003; Piironen, Toivo, & Lampi, 2002; Ross, Shepherd, Schüpphaus, Sinclair, Alfaro, Kamal-Eldin, et al., 2003).

The direct analysis of these minor compounds is often challenging due to their low concentration in cereals (often less than 0.1 mg/g) and the complex mixture of concomitant lipids potentially coeluting with them. Recently, Prinsen et al. analysed lipids from wheat bran by gas chromatography coupled to flame ionisation detection and mass spectrometry (GC-FID and GC-MS) using short and medium length columns (5 and 15 m). While a large number of compounds could be identified, many coelutions resulting from sample complexity, could be observed (Prinsen, Gutierrez, Faulds, & del Rio, 2014). To mitigate this issue, different approaches have been proposed. Alkaline saponification of the lipids, as frequently employed for sterol analysis, can be used to hydrolyse all ester bonds, leading to a simplification of the lipid spectrum (Cert, Moreda, & Pérez-Camino, 2000). Subsequently, the free fatty acids can be easily separated, effectively enriching unsaponifiable compounds such as sterols or aliphatic alcohols (Cert, Moreda, & Pérez-Camino, 2000). However, this step is laborious and leads to a loss of information about the native distribution of lipids; it can also result in the degradation of lipids (e.g. tocopherols) or the formation of artefacts (Abidi, 2001; Rupérez, Martín, Herrera, & Barbas, 2001).

A second approach is the selective isolation of chosen lipid classes of interest from the total lipid extract (TLE) by chromatographic methods prior to analysis (Ruiz-Gutierrez & Perez-Camino, 2000). While well-established and effective, these methods similarly only allow for certain lipids to be investigated (loss of information). By employing selective detection techniques, e.g. by coupling high performance liquid chromatography to fluorescence detection (HPLC-FLD) or using LC-MS or GC-MS in the selected ion monitoring or selective reaction monitoring modes, pre-selected

compounds can be detected with high sensitivity and selectivity, although again with the caveat of being restricted to a few chosen targets.

One way to mitigate these drawbacks is through optimisation of the analytical system to allow the separation, detection and identification of an increased number of compounds. The two major strategies to achieve this are to extend the elution window (e.g. through higher GC operation temperatures) or by increasing selectivity, particularly through multidimensional chromatographic approaches. Comprehensive LC x GC and GC x GC coupled to mass spectrometry were indeed used to great effect for the detailed investigation of total lipid patterns and the parallel screening of multiple classes of minor lipids (Janssen, de Koning, & Brinkman, 2004; Purcaro, Barp, Beccaria, & Conte, 2016).

In this study we combined gas chromatography and high resolution quadrupole-time-of-flight mass spectrometry (GC/Q-TOF MS) for the profiling of GC-amenable lipids in cereals. By extending the GC elution window to 350 °C TLEs could be analysed without any prior hydrolysis step, therefore preserving the original lipid composition. Through the acquisition of high resolution full-scan mass spectrometry data, information on multiple compound classes (with the increased selectivity and certainty of identification provided by high mass accuracy) could be obtained simultaneously thereby retaining the capability to retrospectively inspect data for other novel compounds of interest. Recently, this method was used to profile cereal lipids to detect and identify cereal lipid biomarkers to be used in archaeological research (Hammann & Cramp, 2018). Here we use this new approach to analyse the lipids from 77 samples of bread wheat, spelt, einkorn, emmer, barley, rye and oats and compare their lipid profiles with a particular focus on the lipids present at low concentration. In addition, the study aimed to investigate whether the distributions of the GC-amenable

lipids could be used to discriminate between the seven cereal classes investigated in this study and be used to test the authenticity of cereals.

2. Materials and Methods

2.1 Chemicals

n-Hexane, dichloromethane (DCM), chloroform and methanol (all HPLC grade) were purchased from Rathburn Chemicals (Walkerburn, UK), while the silylating agent BSTFA/TMCS 99:1 (v/v) was purchased from Supelco (Bellefonte, PA, USA). Pyridine (>99%), palmitic acid, methyl heptadecanoate (17:0-ME), methyl stearate, stearic acid, cholesterol, 1-palmitoyl-glycerol, dipalmitoyl-glycerol, trimyristate, tripalmitate, tristearate (all >99%), tetratriacontane (*n*-C₃₄, 98%), and silica were purchased from Sigma-Aldrich (Irvine, UK). Cholesteryl oleate (97%) was purchased from Lancaster Synthesis (Morecambe, UK) and an authentic standard of 5-*n*-docosylresorcinol (AR-22, >99%) was purchased from ReseaChem (Burgdorf, Switzerland). All glass consumables (test tubes, pipettes, vials, chromatography columns) were heated in a muffle furnace at 450 °C for 4h prior to use.

2.2 Samples

Grain samples of bread wheat (*Triticum aestivum*, n=14), einkorn (*Triticum monococcum*, n=11), emmer (*Triticum dicoccum*, n=14), spelt (*Triticum spelta*, n=10), barley (*Hordeum vulgare*, n=11), oats (*Avena sativa*) and rye (*Secale cereale*, n=8) were sourced from collections of collaborating researchers, the Max Rubner Institute

(Detmold, Germany), the John Innes Centre (Norwich, UK) (Horler, Turner, Fretter, & Ambrose, 2018), and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany). The varieties chosen for analysis were predominantly local landraces from Europe. A detailed sample list can be found in Table S1 (Supporting information).

2.3 Extraction of cereals

Lipid extraction was performed as described previously (Hammann & Cramp, 2018). In short, cereals were ground using a mortar and pestle and the lipids were extracted using chloroform/methanol 2:1 (v/v) (2x10 mL) and *n*-hexane (10 mL).

Aliquots of the TLEs were cleaned on a small chromatography column (1 cm i. d.) to remove GC-unamenable polar lipids such as glycerophospholipids. The column was filled with 0.5 g activated silica and conditioned with 5 mL chloroform/methanol 2:1 (v/v) before sample solutions were applied and eluted with 5 mL chloroform/methanol 2:1 (v/v). The eluate was pipetted into a 1.75 mL vial and the solvent was removed under a gentle stream of nitrogen. Afterwards, the residue was re-dissolved in 1 mL chloroform/methanol 2:1 (v/v).

2.4 Derivatisation

Pyridine (25 µL) and 50 µL of the silylating agent (BSTFA/TMCS 99:1, v/v) were added to a dried aliquot of the silica-cleaned TLE (250 µL), the vial was tightly sealed and heated at 70 °C for 1h. After cooling, pyridine and excess derivatisation agent were removed under a gentle stream of nitrogen, and the residue was re-dissolved in

0.5 mL of *n*-hexane. After addition of 6 µg of the second internal standard, methyl heptadecanoate, this solution was analysed by GC-FID and GC/Q-TOF MS.

2.5 GC-FID analysis of trimethylsilylated lipid extracts

Trimethylsilylated TLEs were analysed using a 7890 GC-FID instrument (Agilent, Santa Clara, CA/USA) as described previously (Hammann & Cramp, 2018). A 15 m, 0.32 mm i.d., 0.1 µm film thickness DB-1HT column (100 % dimethylpolysiloxane, Agilent, Santa Clara, CA/USA) was used for the separation, and injections (1 µL) were made using a 7683B autosampler *via* a cool-on-column inlet. Helium was used as the carrier gas at a constant flow rate of 4.0 mL/min. The GC temperature program was as follows: After 2 minutes at 50 °C, the temperature was raised at a rate of 10 °C/min to 350 °C (held for 10 min). The temperature of the inlet was set to track the oven temperature and the FID temperature was set to 350 °C while flow rates of hydrogen, air and nitrogen were set to 30, 400 and 27 mL/min, respectively.

2.6 GC/Q-TOF MS analysis of trimethylsilylated lipid extracts

Subsequent to GC-FID analysis, TLEs were analysed by a 7890 GC coupled to a 7200B GC/Q-TOF MS (Agilent, Santa Clara, CA, USA). Injections (1 µL) were made using 7693 autosampler and a cool-on-column inlet, which was set to follow the oven temperature. A 15 m, 0.25 mm i.d., 0.1 µm film thickness ZB-5HT Inferno column (Phenomenex, Torrance, CA, USA) was installed in the GC oven. Helium was used as the carrier gas at a flow rate of 1.5 mL/min and the GC oven was programmed as

follows: After 2 min at 55 °C the temperature was increased to 220 °C at a rate of 10 °C/min followed by an increase to 350 °C at 20 °C/min (held for 13 minutes).

The temperatures of the ion source, quadrupole and transfer line were set to 300 °C, 180 °C and 350 °C, respectively. Data were acquired, following a solvent delay of six minutes, in full scan mode from m/z 50-1050 with a sample rate of 5 spectra/s using the *Extended Dynamic Range* mode. A standard mix consisting of palmitic acid, stearic acid, methyl heptadecanoate, methyl stearate, 1-palmitoyl-glycerol, cholesterol, tetratriacontane, dipalmitoyl-glycerol, trimyristate, cholesteryl oleate, tripalmitate, and tristearate was analysed (after silylation) with every standard batch to ascertain chromatographic and mass spectrometric performance.

GC/Q-TOF MS data were analysed using MassHunter Qual (Version B.07.00), and MassProfiler Pro (Version B.14.5) (both Agilent, Santa Clara, CA, USA) and further processed using a MZmine 2 workflow (see section 2.7).

2.7. Data processing workflow for statistical analysis

An optimized MZmine 2 (version 2.36) workflow has been developed to generate an aligned feature list for statistical analysis (Pluskal, Castillo, Villar-Briones, & Orešič, 2010). A feature can be perceived as a row of a matrix, which describes a peak in retention time dimension with a corresponding m/z value. This matrix is called a feature list. An aligned feature list consists of multiple feature lists, whose features have been compared and if found equal with respect to a certain m/z and retention time window are aligned to the same matrix row. Prior to the data processing itself, raw data conversion was applied to the open format mzML using MSConvert to enable MZmine 2 compatibility (Chambers, Maclean, Burke, Amodei, Ruderman, Neumann,

et al., 2012). The MZmine 2 workflow contained the recursive threshold mass detection algorithm, with a noise level set to 100 for the first 20 minutes and 500 for the time window of 20 - 38.5 minutes. The minimum peak m/z width was set to 0.05 and the maximum peak m/z width was set to 1. The mass detection was separated in two groups with different noise levels, due to a significantly higher baseline, starting at approximately 20 min. The resulting mass lists were used for chromatogram building, which included the parameter *minimum time span*, which was set to 0.02 minutes. The value sets the minimum time for which the same ion must be observed to be recognized as a chromatogram. A minimum peak intensity was set to 1000 and the relative mass tolerance was set to 5 ppm. All chromatograms were smoothed over 13 data points. The next step was chromatogram deconvolution by applying the local minimum search algorithm, including a chromatographic threshold of 90%, a minimum peak top/edge ratio of 2, a minimum peak intensity set to 1000 and a maximum peak duration of 2 minutes. To further reduce the size of the data, isotopic features were removed by applying the isotopic peaks grouper algorithm. The parameters for the algorithm were set to a relative mass accuracy of 5 ppm, a retention time tolerance of 0.05 minutes and the maximum allowed charge was set to 1. To deal with retention time shifts occurring in the large data set, mainly caused by column maintenance (trimming), retention time normalization was performed using the MZmine 2 retention time normaliser algorithm with the parameters 20 ppm as m/z tolerance, 1 minute as retention time tolerance and 1000 as minimum intensity of the peak selected for normalisation. All feature lists were then aligned using the join aligner algorithm with m/z tolerance set to 25 ppm and retention time tolerance was set to 0.5 min. To fill in missing values of the aligned feature list, the "same m/z and RT range filler" gap filling algorithm was applied with an m/z tolerance of 20 ppm. The resulting aligned feature

list was exported with the *Export to MetaboAnalyst* file method. The exported .csv file was uploaded to the MetaboAnalyst 4.0 web server for further data processing (Chong, Soufan, Li, Caraus, Li, Bourque, et al., 2018). The data was uploaded as *Peak intensity table* with *Samples in columns (unpaired)* format and was normalized using the generalized logarithm (glog) transformation. The final matrix was processed for Principle Component Analysis (PCA) and Random Forest feature search using MetaboAnalyst 4.0.

3. Results and discussion

3.1 Lipid analysis by GC-FID and GC/Q-TOF MS

3.1.1 Analysis of cereal lipid extracts by GC-FID and contents of GC-amenable lipids in cereal samples

With our set-up we were able to analyse GC-amenable lipids without prior hydrolysis. The first lipid compounds to elute were usually free fatty acids, followed by monoacylglycerols, alkylresorcinols, tocots and free sterols. This was followed by the elution of diacylglycerides and conjugated sterols (sterol fatty acid esters and sterol glycosides) before finally triacylglycerols (TAGs, usually C₄₈ to C₅₆ and, rarely, C₅₈) eluted (. This means that compounds up to a molecular weight of about 900 Da could be eluted and analysed at a maximum GC oven temperature of 350 °C. This was deemed appropriate since TAGs are generally the least volatile lipids of interest.

The highest lipid contents (as determined by GC-FID) were observed in oat samples (median 38.2 mg/g dry matter), which was significantly higher than in the other cereals (median values of 7.2 – 16.9 mg/g dry matter) (Table S2). It should be noted that with this method we could only detect GC-amenable lipids, which excluded

some abundant polar lipids such as glycerophospholipids that were removed during sample preparation (section 2.3). Furthermore, the lipid extracts also contained some non-lipid compounds, i.e. peaks relating to saccharide structures.

3.1.2 Analysis of cereal lipid extracts by GC/Q-TOF MS

Despite the slightly higher polarity of the GC stationary phase in GC/Q-TOF MS the general elution pattern was the same as in GC-FID analysis (Figure 2a) and mass spectra for several hundred individual compounds in the samples could be acquired. Furthermore, extracted ion chromatograms, using the accurate mass of individual compounds (e.g. m/z 486.4257 for the molecular ion of trimethylsilylated sitosterol) or compound classes (e.g. m/z 268.1315 for bis-trimethylsilylated alkylresorcinols) with an extraction window of ± 10 ppm, facilitated searching and detecting compounds of interest, and determining distributions of compounds that could then be compared between samples. Importantly, non-targeted peak-picking and deconvolution workflows could be used on the dataset to find unknown compounds and those not readily detected due to co-elution (see sections 2.7 and 3.5). Although identification without corresponding reference compounds can be difficult, the number of detected (and potentially identifiable) compounds was greatly increased using this approach. In the following sections we will specifically focus on the data on the patterns of sterols (section 3.2), tocopherols, tocomonoenols, tocotrienols (section 3.3), and alkylresorcinols (section 3.4) in the cereal samples.

3.2. Free and conjugated sterols detected by GC/Q-TOF MS in the cereal lipid extracts

Cereals are an important source of dietary phytosterols in human nutrition (Piironen, Toivo, & Lampi, 2002). Phytosterols have been attributed with cholesterol-lowering effects in humans and are therefore, in terms of nutrition, particularly desirable components of cereals. Critically, the bioavailability and physiological effects in humans can vary between free and conjugated sterols (Nyström, Moreau, Lampi, Hicks, & Piironen, 2008).

Sterols could be detected as free sterols, esterified to fatty acids and ferulic acid, and as steryl glucosides (Figure 1a-d). The first sterol compounds to elute from the GC were the free sterols, followed by glucosides, and finally fatty acid (C₁₆ and C₁₈) and ferulic acid esters (Figure 2b, Table 1). In the free sterol fraction, up to 10 sterols could be identified. The main sterols identified (See Table S3, supporting information) were sitosterol and campesterol alongside their corresponding saturated stanol analogues, i.e. stigmastanol and campestanol (Piironen, Toivo, & Lampi, 2002). Further minor sterols included stigmasterol, Δ^5 -avenasterol and 24-methylene-cycloartanol. A few samples exhibited a prominent peak eluting shortly before campesterol, which was identified as ergosterol. Ergosterol is the predominant sterol in several fungi and the detection of ergosterol could mean that these particular samples had been infected (Perkowski, Buśko, Stuper, Kostecki, Matysiak, & Sz wajkowska-Michalek, 2008; Varga, Bartók, & Mesterházy, 2006). This demonstrates the added-value of this method as a quality screening tool for contamination and spoilage.

In the conjugated sterol fraction, C₁₆ and C₁₈ fatty acid esters of campesterol, campestanol, stigmasterol, sitosterol, stigmastanol, and Δ^5 -avenasterol were detected in all samples, but in varying compositions. Whilst C₁₈ esters dominated over C₁₆ esters in most bread wheat samples, as well as einkorn, emmer and oats, they were

about the same intensity in rye; finally, barley and spelt samples contained higher relative concentrations of the C₁₆ esters (Table 1). Owing to the non-polar nature of the stationary phase, no separation of the esters with degrees of fatty acid unsaturation could be achieved and potential differences in the C₁₈ fatty acid composition could not be observed. In addition to fatty acid esters, ferulic acid esters of campestanol and stigmastanol were also detectable at low concentrations in samples of bread wheat, einkorn, emmer, spelt, and rye. Campestanol esters were more abundant than the corresponding stigmastanol esters (Figure S1, supporting Information). The esters eluted as two sets of peaks, with the earlier eluting cis-isomers being less abundant than the later eluting trans isomers. The relatively high abundance of the cis-isomers was likely due to isomerisation from light during sample preparation (Esche, Barnsteiner, Scholz, & Engel, 2012).

Finally, campesterol, campestanol, stigmasterol, sitosterol, stigmastanol, and Δ^5 -avenasterol could also be detected as glycosides and identified based on their mass spectra (Gutiérrez & del Río, 2001) (Table 1, Table S3, supporting information). Furthermore, eluting very late, together with triacylglycerides, peaks tentatively identified as acylated sterol glycosides were detected. However, due to their late elution and low concentrations no unequivocal mass spectra could be gathered.

3.3 Tocopherols, tocotrienols and tocomonoenols detected by GC/Q-TOF MS in bread wheat, einkorn, emmer, spelt, barley, rye, and oats

Tocopherols and tocotrienols, although only present at total levels of <0.1 mg/g could be readily detected and identified from their characteristic mass spectra, which enabled determination of the number of methyl groups on the 6-chromanol ring, as

well as the number of double bonds in the isoprenic side chain. For example, α -tocopherol (Figure 1e) yielded a molecular ion (as its TMS ester) at m/z 502.4195 (-2.2 ppm) and the chromanol ring-characteristic tropylium fragment-ion at m/z 237.1289 (-9.3 ppm). In contrast, in the mass spectrum of α -tocotrienol the molecular ion was detected at m/z 496.3715 (corresponding to a difference of 6 hydrogen atoms or 3 double bonds, -4.4 ppm), whilst γ -tocopherol was identified by the molecular ion at m/z 488.4037 (-2.6 ppm) and the tropylium fragment ion at m/z 223.1146 (corresponding to the substitution of one methyl group by a hydrogen, -4.0 ppm). It should be noted that β - and γ - only differ in the substitution pattern and are not readily resolved by the GC column used in this study. They also have very similar mass spectra, but we noted a higher intensity of the fragment ion at m/z 222.1076 over m/z 223.1155 in the GC/Q-TOF mass spectra of β/γ -T in all cereals but not in barley. Of all the investigated cereals, only barley has been reported to contain predominantly the γ -isomer, while in the other cereals the β -isomer was dominant. This fragmentation behaviour could be confirmed with authentic standards of β - and γ -tocopherol, and while it does not allow to accurately quantify the contributions of β - and γ -isomers it could potentially allow the qualitative estimation of the dominant isomer.

In total up to 6 distinct tocol peaks could be detected in the samples, but the distributions differed for the cereal classes. While the tocol distribution in rye samples was dominated by α -tocopherol, einkorn samples (as shown in Figure 2c) exhibited β/γ -tocotrienol as the most abundant isomer. The distributions in the cereals used for this study were not fully consistent with previous literature reports (Panfili, Fratianni, & Irano, 2003; Ryyänänen, Lampi, Salo-Väänänen, Ollilainen, & Piironen, 2004). While the ratios of α -tocopherol to β/γ -tocopherol are consistent with literature reports, markedly lower contributions of the corresponding tocotrienols were found in this

study. Tocotrienols are particularly liable to oxidation and degradation and the sample preparation protocol used was not optimised to mitigate this (Rupérez, Martín, Herrera, & Barbas, 2001). The addition of antioxidants such as butylated hydroxytoluene to the extraction solutions might be a way to fully retain the original vitamin E distribution in future research.

In addition to tocopherols and tocotrienols α - and β - γ -tocomonoenols (Figure 1f) were also detected at concentrations of about 1-2% of the corresponding tocopherols (Figure 2c). These monounsaturated tocopherol-derivatives, having only recently come into scientific focus, have been detected in a few matrices, but little is yet known about their occurrence and physiological role (Butinar, Bucar-Miklavcic, Mariani, & Raspor, 2011; Fiorentino, Mastellone, D'Abrosca, Pacifico, Scognamiglio, Cefarelli, et al., 2009; Müller, Hammann, & Vetter, 2018). These minor compounds eluted slightly after the corresponding tocopherols, and could be identified by their mass spectra, i.e. the tropylium fragment ion at m/z 237.1328 (+7.2 ppm) and the molecular ion at m/z 500.4024 (-5.0 ppm) for α -tocomonoenol. As far as can be ascertained, this is the first report of the presence of these compounds in cereals.

3.4 Alkylresorcinol distributions in bread wheat, einkorn, emmer, spelt, barley, rye, and oats

Alkylresorcinols (ARs) are a class of compounds that share a common resorcinol backbone with an alkyl substituent at the *meta*-position (Figure 1h). Through alkyl chains of different carbon numbers several homologues are possible, and they have been frequently detected in cereals such as bread wheat, spelt, rye or barley

(Landberg, Kamal-Eldin, Salmenkallio-Marttila, Rouau, & Åman, 2008; Ross, Åman, Andersson, & Kamal-Eldin, 2004; Ross, Kamal-Eldin, Jung, Shepherd, & Åman, 2001; Ross, et al., 2003; Ziegler, Steingass, Longin, Würschum, Carle, & Schweiggert, 2015).

Alkylresorcinols were detected at appreciable quantities in all cereals but oats, which only contained traces (Figure 3). In general, ARs with chain-lengths of 15–27 carbon atoms (predominantly odd-numbered isomers) were detectable. In addition to the alkylresorcinols the corresponding unsaturated alkenylresorcinols, with a double bond in the alkyl chain, could be detected. These alkenylresorcinols eluted slightly earlier from the GC than the corresponding saturated homologue and could be identified by a combination of the base ion at m/z 268.1315 and the molecular ion which was shifted to a lower mass by 2.0157 Da (corresponding to two hydrogens or one double bond) compared to the respective saturated isomer (e.g. the molecular ions for the bis-TMS derivatives of AR-21 and AR-21:1 were detected at m/z 548.4455 (+1.8 ppm) and 546.4263 (-4.7 ppm), respectively). The alkenylresorcinols were particularly abundant in rye (about 10% of the corresponding saturated analogue) as previously reported by Ross et al. and Suzuki et al. (Ross, et al., 2003; Suzuki, Esumi, Uramoto, Kono, & Sakurai, 1997).

The distribution and pattern of AR homologues differed markedly between the different cereals (Table 2, Table S4). For example, barley samples exhibited AR-25 as the major AR component; this was unique in the cereals investigated. In contrast, all the *Triticum* species (bread wheat, spelt, einkorn and emmer) exhibited AR-21 as the main component, whilst the most abundant AR in rye was AR-19. In oats, only minute traces of AR-19 and AR-21 could be detected with this general absence of ARs being in accordance with the literature (Ross, et al., 2003). It should be noted that AR

distributions did not only vary between cereal classes but also within one class. In emmer the contributions of AR-19 and AR-25 to the total AR content varied between 5 and 25% and 2 and 10%, respectively (Table S4). This demonstrated that different varieties and cultivation conditions could affect the concentration and relative distribution of ARs (Andersson, Kamal-Eldin, & Åman, 2010).

In general, our results for the AR composition were in-line with previously reported values for the cereals investigated here (Hengtrakul, Lorenz, & Mathias, 1991; Landberg, Kamal-Eldin, Salmenkallio-Marttila, Rouau, & Åman, 2008; Ross, Kamal-Eldin, Jung, Shepherd, & Åman, 2001; Ross, et al., 2003; Żarnowski, Suzuki, Yamaguchi, & Pietr, 2002; Ziegler, Steingass, Longin, Würschum, Carle, & Schweiggert, 2015).

3.4.1 Further minor alkylresorcinols detected by GC/Q-TOF MS

In addition to the aforementioned monounsaturated alkenylresorcinols, further minor compounds which contributed even less ($<<0.1\%$) to the AR distribution were also detected. Rye and spelt samples contained trace amounts of di-unsaturated alkenylresorcinols which eluted slightly earlier than the respective monounsaturated and saturated homologues (Figure S2a). Furthermore, traces of even-chain alkylresorcinols (e.g. AR-20 or AR-22) were frequently detected in the samples, eluting exactly between the more abundant odd-chain AR homologues (Figure S2b). These compounds have not been reported in cereals before but have very recently been detected by Ross et al. in quinoa (Ross, Svelander, Karlsson, & Savolainen, 2017). In light of the results from this study, they appear not be restricted to this pseudocereal but are likely more widely distributed. Finally, methyl-alkylresorcinols (mAR), which

feature an additional methyl group on the aromatic ring, were detected; these compounds have also been detected previously in quinoa (Ross, Svelander, Karlsson, & Savolainen, 2017). The mass spectra of these compounds featured a base ion at m/z 282.1472 instead of m/z 268.1315 (i.e. shifted by the mass corresponding to one methylene group compared to ARs). The mAR eluted slightly later than the respective AR isomers with the same alkyl chain and could be identified through the presence of m/z 282.1472 and the respective molecular ion (Figure S2b-d). Various mAR homologues from mAR-19 to mAR-27 could be identified in spelt, bread wheat, barley and emmer, but not in rye, einkorn and oats (which was very low in alkylresorcinols in general). The detection of these compounds in several of the cereal samples (although at minute concentrations) means that the use of these compounds as specific markers for quinoa or – in human plasma – for dietary quinoa intake should be treated with caution.

3.5 Analytical discrimination of bread wheat, spelt, einkorn, emmer, barley, rye and oats by Principle Component Analysis

The lipids and their intensities obtained from the data mining workflow (section 2.7) for most samples (including analytical duplicates from extraction and GC-MS) were compared by PCA. Some samples of bread wheat, barley, emmer and einkorn had been analysed under slightly different conditions and had to be excluded from the PCA (see Table S1, supporting information). With the PCA based on all mass features a good separation of the lipid classes was achieved, particularly when PC1 was plotted against PC3 (Figure S3, supporting information), but a slight overlap between cereals (particularly emmer and einkorn) was observed. However, this separation could be

significantly improved by applying a Random Forest algorithm to identify the most diagnostic mass features. PCA analysis of a dataset reduced to the 25 most impactful classifiers showed a full separation of all seven cereal classes (Figure 4). The first two principle components accounted for 64.7% and 15.8% of total variance, respectively. Rye samples were separated from the other cereals by strongly negative scores in PC1, followed by spelt samples (slightly negative). Oats samples were clearly separated from the other cereals by their strong positive scores in PC1, while bread wheat samples featured clearly more negative scores in PC2 than the other cereals. Einkorn, emmer and barley samples plotted more closely together, but were fully separated by slight differences in both PC1 and PC2. It has to be noted here, that our samples included a high proportion of local landraces, which exhibit higher genetic diversity than modern, inter-bred cereal varieties (Villa, Maxted, Scholten, & Ford-Lloyd, 2005). This diversity is likely expressed in a higher variation in their lipid patterns and therefore in relatively high intra-group variation. The Loadings Plot supported the findings described in the prior results sections (Figure S4, supporting information). Alkylresorcinols (red circles) were found to have predominantly negative loadings on PC1 and PC2, which explains the strongly negative values of rye (high in ARs) samples in PC1 and positive values of oats (low in ARs in both PC1 and PC2). The most negative loading on PC2 was found for a mass feature corresponding to AR-17, and the higher proportions of AR17 in bread wheat compared to barley, emmer, and einkorn explained the separation of these groups on PC2.

A second cluster of compounds (blue circles) in the loadings plot was found with slightly positive loadings on PC1 and mostly slightly negative loadings on PC2. These mass features were found to correspond to triacylglycerols in the samples. In addition to ARs and triacylglycerols, there were few compounds (green circles) which

could not readily be identified, but their corresponding mass spectra tentatively suggested the presence of at least one carbohydrate moiety in their structure.

Ziegler et al. reported an analytical separation of *Triticum* ssp. by PCA based on relative AR distribution, and were able to achieve a separation of einkorn (diploid) from emmer and durum wheat (both tetraploid) and spelt and bread wheat (both hexaploid), but the two hexaploid wheats were not separable (Ziegler, Steingass, Longin, Würschum, Carle, & Schweiggert, 2015). Righetti et al. achieved a separation of einkorn, emmer, and spelt as well as durum and bread wheat by PCA based on non-targeted lipid profiles acquired by high resolution LC-MS (Righetti, Rubert, Galaverna, Folloni, Ranieri, Stranska-Zachariasova, et al., 2016; Righetti, Rubert, Galaverna, Hurkova, Dall'Asta, Hajslova, et al., 2018). As far as can be ascertained, this study is the first to successfully discriminate seven cereals, including four closely related *Triticum* species, based on their lipid profiles. Based on this, GC/Q-TOF MS-based lipid profiling could be used as an authenticity tool to distinguish cereal flours or guide the selection of specific marker compounds and derived methods to be used to detect mixtures.

3.6 Limitations of the approach

One drawback of our GC-based approach is the exclusion of polar lipids such as glycerophospholipids or digalactosyldiacylglycerols, which represent important lipid classes in cereals (Geng, Harnly, & Chen, 2015; Morrison, 1988). The analysis of these compounds can be achieved by LC-MS, but this technique has inherent caveats due to vastly different ionisation efficiencies of different analytes depending both on ionisation technique (e.g. APCI or ESI) and polarity (positive or negative). In reference

experiments, LC-MS with ESI in positive mode was found to be very useful for the analysis of phospholipids, but alkylresorcinols were not detectable using this approach and required use of APCI or negative ionisation ESI (Knödler, Berardini, Kammerer, Carle, & Schieber, 2007; Righetti, et al., 2016). Accordingly, lipid profiling by LC-MS is not straightforward; both GC- and LC-based methods have their individual advantages and weaknesses.

Compared with analytical approaches employing targeted sample preparation steps, the approach used in this study could be slightly less sensitive because dominant lipids (mostly TAGs) do not allow the same concentration factor for analysis. Our instrumental limit of detection was about 1 pg on column (corresponding to about 50 ng/g cereal) for alkylresorcinols and free sterols, but slightly higher for later eluting compounds such as the late eluting C₅₄ TAGs (20 pg and 1 µg/g cereal, respectively). Accordingly, we were only able to detect major compounds in the conjugated sterol fraction (typically conjugates of sitosterol and campesterol), whilst other studies have provided a more detailed analysis (Esche, Barnsteiner, Scholz, & Engel, 2012). However, this analytical approach enabled individual detection of all these conjugates in one analytical run without requiring dedicated sample preparation steps for each of these fractions (Breinhölder, Mosca, & Lindner, 2002). Furthermore, novel minor constituents of the vitamin E fraction (i.e. tocomonoenols) and minor alkylresorcinols (i.e. even-chain ARs and mARs) which had not been described in cereals before were detectable using this approach, demonstrating the sensitivity achievable with this non-targeted approach.

4. Conclusions

A new analytical approach for lipid profiling, based on high temperature gas chromatography coupled to high resolution mass spectrometry, was successfully applied to the analysis of cereal lipids. Whilst gathering analytical data on the several hundred compounds of the complete GC-amenable lipidome the method proved to be sensitive enough for the detection of nutritionally or analytically important minor compounds, such as free and conjugated sterols and alkylresorcinols. Furthermore, distinct differences in the distribution in the lipid patterns could be used to discriminate all seven cereals by PCA, thus proving the value of this method as authenticity tool.

The method could be further developed and improved by using compound class-specific standards to account for losses during sample preparation and to enable quantitative determination of the lipid constituents. This could provide a valid alternative to LC-MS based lipid-profiling methods, to be used for other matrices or as an addition to an LC-MS assay of polar lipids.

Conflict of interests:

The authors have no conflicts of interests.

Acknowledgements:

The authors are grateful to A. Bogaard (University of Oxford, UK), F. Longin (University of Hohenheim, Germany), the Max Rubner Institute (Detmold, Germany), the John Innes Centre (Norwich, UK), and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany) for cereal samples. S.H. and L.J.E.C. were supported by the Royal Society (RG150718) and NERC (NE/N011317/1). GC/Q-ToF

MS (CC010NERC capital grant awarded to IDB) analyses were performed at the NERC Life Sciences Mass Spectrometry Facility (Bristol Node). The authors thank the Natural Environment Research Council, UK for partial funding of the mass spectrometry facilities at Bristol (Contract No. R8/H10/63; <http://www.lsmsf.co.uk>).

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Captions to Figures:

Figure 1: Structures of a) sitosterol, b) sitosteryl palmitate, c) stigmastanyl ferulate, d) sitosteryl glucoside, e) α -tocopherol, f) α -tocomonoenol, g) α -tocotrienol, and h) 5-*n*-nonadecyl resorcinol (AR-19).

Figure 2: a) GC/Q-TOF MS chromatogram of a representative lipid extract, after trimethylsilylation, showing the elution of fatty acids, alkylresorcinols, tocols, free and conjugated sterols as well as diacylglycerols (DAGs) and finally triacylglycerols (TAGs). Peaks marked with asterisks are internal standards. b): Partial extracted ion chromatograms of m/z 396.3756 (black), m/z 397.3834 (red), m/z 382.3600 (orange), and m/z 383.3678 (blue) showing the elution of free sterols as well as steryl glycosides and C₁₆ and C₁₈ fatty acid esters. c): Partial extracted ion chromatograms showing the elution of β/γ -tocopherol (T, m/z 488.4050, dark blue), β/γ -tocomonoenol (T1, m/z 486.3894, green), β/γ -tocotrienol (T3, m/z 482.3581, red), α -tocopherol (m/z 502.4206, black), α -tocomonoenol (m/z 500.4050 orange), and α -tocotrienol (m/z 496.3737, light blue). The peaks marked with a plus are sitosterol and stigmastanol.

Figure 3: Partial extracted ion chromatograms of m/z 268.1315 showing the elution of the major alkylresorcinols and alkenylresorcinols with chain lengths from 15–27 carbons in representative samples of rye, bread wheat, spelt, einkorn, emmer, barley and oats. Please note: Individual chromatograms were slightly shifted on the x axis (\leq 0.2 min) to account for retention time shifts from column maintenance.

Figure 4: Score plot of the Principal Component Analysis of the cereal samples (including duplicates) based on a reduced feature list showing the separation of bread wheat (green), einkorn (blue), emmer (turquoise), spelt (grey), barley (red), rye (yellow) and oats (pink). A Random Forest classification algorithm (Metaboanalyst) was applied to the total lipid profiles extracted from the samples and the 25 most impactful features were selected for the reduced feature list.